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Fate of 3,3'-Dichlorobenzidine in Aquatic Environments

Syracuse Research Corp, NY Life Sciences Div

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Several aspects of the aquatic environmental fate of 3,3'-dichlorobenzidine (DCB), a suspected human carcinogen, were examined. Greater than 95% of dichlorobenzidine present was adsorbed to natural pond and lake sediments in aqueous suspensions. Only a portion of the adsorbed chemical could be extracted from the sediments, with this amount decreasing over time, suggesting chemical reaction of DCB with sediment constituents. Dichlorobenzidine was rapidly degraded by natural and artificial light in aqueous solution, with a half-life of the order of 90 seconds in natural sunlight. Monochlorobenzidine and benzidine were found to be intermediate products of this process. In contrast, DCB appeared recalcitrant to degradation by naturally occurring aquatic microbial communities with only a minor loss of chemical detected over a 30-day incubation period. Dichlorobenzidine was rapidly bioconcentrated in bluegill sunfish, with mortality occurring prior to establishment of a chemical equilibrium between water and fish. Bioconcentration factors of 132-554 were achieved at this point. The only metabolite detected in the fish was an acid-labile conjugate of DCB. Based on these observations, chemical and physical processes, rather than biological ones, appear to be the important factors governing the fate of DCB in the aquatic environment. The ability of DCB to concentration in aquatic organisms may pose a direct hazard to human health through consumption of contaminated fish.

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FATE OF 3,3'-DICHLOROBENZIDINE IN AQUATIC ENVIRONMENTS

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FOREWORD

Environmental protection efforts are increasingly directed towards prevention of adverse health and ecolgical effects associated with specific compounds of natural or human origin. As part of this Laboratory's research on the occurrence, movement, transformation, impact, and control of environmental contaminants, the Environmental Processes Branch studies the microbiological, chemical, and physico-chemical processes that control the transport, transformation, and impact of pollutants in soil and water.

The discharge of 3,3'-dichlorobenzidine (DCB), which is regarded as a human carcinogen, into the aquatic environment is of great concern to human health because of possible exposure through drinking contaminated water or eating fish in which the chemical or its metabolites have accumulated. The potential hazard of the chemical may be compounded by its biological or non-biological conversion to compounds of even greater toxicity or persistence than the parent chemical. This study assesses the role of several environmental processes affecting the fate of the chemical in natural waters to provide information needed for establishing effluent guidelines for DCB.

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ABSTRACT

Several aspects of the aquatic environmental fate of 3,3'-dichlorobenzidine (DCB), a suspected human carcinogen, were examined. Greater than 95% of dichlorobenzidine present was adsorbed to natural pond and lake sediments in aqueous suspensions. Only a portion of the adsorbed chemical could be extracted from the sediments, with this amount decreasing over time, suggesting chemical reaction of DCB with sediment constituents. Dichlorobenzidine was rapidly degraded by natural and artificial light in aqueous solution, with a half-life of the order of 90 seconds in natural sunlight. Monochlorobenzidine and benzidine were found to be intermediate products of this process. In contrast, DCB appeared recalcitrant to degradation by naturally occurring aquatic microbial communities with only a minor loss of chemical detected over a 30-day incubation period. Dichlorobenzidine was rapidly bioconcentrated in bluegill sunfish, with mortality occurring prior to establishment of a chemical equilibrium between water and fish. Bioconcentration factors of 132-554 were achieved at this point. The only metabolite detected in the fish was an acid-labile conjugate of DCB. Based on these observations, chemical and physical processes, rather than biological ones, appear to be the important factors governing the fate of DCB in the aquatic environment. The ability of DCB to concentrate in aquatic organisms may pose a direct hazard to human health through consumption of contaminated fish.

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LIST OF ABBREVIATIONS

DCB --	3,3'-dichlorobenzidine
GC-MS --	gas chromatography-mass spectrometry
GLC --	gas-liquid chromatography
HPLC --	high performance liquid chromatography
MCB --	3-monochlorobenzidine
MLSS --	mixed liquor suspended solids
NMR --	nuclear magnetic resonance
TLC --	thin-layer chromatography

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SECTION 1

INTRODUCTION

3,3'-Dichlorobenzidine (3,3'-dichloro 4,4'-diaminobiphenyl), hereafter referred to as DCB, is widely used as an intermediate in the manufacture of azo pigments. It is of considerable commercial importance; total DCB production in the United States in 1972 was about 4.6 million pounds (1). With current work practices, effluents containing this chemical are discharged directly into receiving waters. Moreover, the discharge of dichlorobenzidine-pigment wastes into receiving waters constitutes an additional source of DCB contamination in the environment since free, unreacted DCB is reported to be present in these pigments.

Dichlorobenzidine is known to induce cancer in animals (2) and is regarded by the Occupational Health and Safety Administration (OSHA) as being carcinogenic to man (3). The discharge of DCB into the aquatic environment is of great concern to human health because of possible exposure to the chemical through drinking-water supplies. Also, DCB and its metabolites may accumulate in fish and could pose a health hazard if fish from contaminated waters were to be used as human food. The potential hazard of DCB may be compounded by its biological or non-biological conversion to compounds of even greater toxicity and/or persistence than the parent chemical. For instance, it is believed that in the case of carcinogenic aromatic amines, it is the metabolites of the chemicals that produce the carcinogenic response (4). Furthermore, potential degradation products of DCB, such as benzidine, may constitute an even greater carcinogenic hazard. Therefore, in order to fully evaluate the hazards associated with the release of DCB into the environment, it becomes important to study the environmental fate of the chemical because its persistence, disappearance, or partial transformation will determine the degree of its hazard.

A number of physical, chemical and biological factors determine the fate of a chemical in the aquatic environment. These include adsorption to sediment, chemical hydrolysis, photodegradation, microbial degradation, and uptake and metabolism by aquatic organisms. Currently nothing is known about the effect of these factors on the persistence and transformation of DCB in the aquatic environment. This study was undertaken to assess the role of some of the processes that may determine the environmental behavior of the chemical. The overall objective of this investigation was to obtain information needed for establishing effluent guidelines for DCB.

Specific Objectives:

1. To determine the sorption-desorption of DCB by aquatic sediments.

2. To study the photodegradation of DCB in simulated and natural sunlight.

3. To study the biodegradation of DCB by microorganisms in lake water and activated sludge.

4. To determine whether aquatic microorganisms can liberate DCB from DCB-based azo dyes.

5. To determine the uptake, elimination, and metabolism of DCB by fish.

SECTION 2

CONCLUSIONS

1. DCB adsorbs extensively to a variety of aquatic sediments and becomes more tightly bound to the sediment with the passage of time.

2. DCB is very rapidly degraded in aqueous solutions by the action of natural or simulated sunlight. 3-Chlorobenzidine (MCB) and benzidine are intermediates generated in this process.

3. DCB is resistant to degradation by naturally occurring aquatic microbial communities.

4. DCB is rapidly bioconcentrated into both the edible and non-edible portions of bluegill sunfish. Mortality occurred prior to establishment of a chemical equilibrium between water and fish with bioconcentration factors of 132-554 achieved at this point. The only DCB metabolite detected in the fish was an acid-labile conjugate of DCB.

SECTION 3

RECOMMENDATIONS

1. Based on available data, DCB, a suspected human carcinogen, should be viewed as a significant hazard to human health and environmental well-being when present in aquatic ecosystems. It has demonstrated properties common to many hazardous environmental contaminants, such as resistance to biodegradation and the ability to bioconcentrate in aquatic organisms. The major pathway of DCB destruction, photodegradation, increases the hazard through generation of benzidine, a carcinogen in humans. The discharge of DCB-containing effluents into water systems should therefore be subject to strict regulation.

2. Studies should be undertaken to determine the ability of DCB to undergo biomagnification through trophic levels. Bioconcentration studies in phytoplankton and zooplankton are also indicated.

3. The toxicity of DCB to aquatic organisms should be studied to determine its adverse effects on aquatic biota.

4. The mechanism of photodegradation should be examined, and the photoproducts should be tested for toxicity.

5. The relative contribution of various mechanisms governing DCB fate (adsorption, photodegradation, biodegradation, and bioconcentration) acting in concert should be studied in model environmental microcosms.

SECTION 4

MATERIAL AND METHODS

CHEMICALS

3,3'-Dichlorobenzidine dihydrochloride (ring-UL-^{14}C)(DCB), 5mCi/mMole, was obtained from California Bionuclear Corp., Sun Valley, CA. Radiochemical purity was judged to be greater than 99% by TLC on silica gel 60 (Merck) in systems of ether:hexane (3:1 v/v) and benzene:ethyl acetate:acetic acid (7:3:0.1 by volume). Only one radioactive peak, chromatographing with non-labeled DCB, was detected upon scanning the developed plates with a Nuclear Chicago Actigraph II.

Non-radioactive recrystallized DCB.2HCl was provided by Fine Chemicals Division of UpJohn Co., North Haven, CT. Under a uv lamp, only one spot was detected on thin-layer chromatograms of DCB developed in the above solvent systems. The dihydrochloride melted in stages between 173-210°C. Because of the absence of published melting point data for the dihydrochloride salt, DCB-free amine was prepared and found to melt at 131-132°C (literature m.p. 132-133°). High performance liquid chromatography did not reveal the presence of benzidine or mono-chlorobenzidine (MCB) in freshly prepared stock solutions of DCB.2HCl. The purity of DCB was further confirmed by NMR and combined GC-MS analyses.

All DCB stock solutions were refrigerated and protected from light during storage. Unless specifically stated, the dihydrochloride of DCB was used in this investigation, and calculations of DCB equivalents were based on the salt form.

Benzidine (dihydrochloride salt) was obtained from J.T. Baker Chemical Co., Philipsburgh, NJ, and 3-chlorobenzidine (MCB) was synthesized according to Branch (5).

All other chemicals used were of reagent grade.

ANALYTICAL METHODS

Thin-Layer Chromatography

All TLC analyses were performed on silica gel 60 (Merck). Non-radioactive DCB and benzidine were visualized under a hand-held uv lamp. Radiographic analyses were performed with a Nuclear Chicago Actigraph II

chromatogram scanner. Systems utilized in this study included: (1) ether: hexane (3:1 v/v)-R_f DCB = 0.37, R_f benzidine = 0.17; (2) benzene:ethyl acetate:gl. acetic acid (7:3:0.1)-R_f DCB = 0.60, R_f benzidine = 0.07.

Gas-liquid Chromatography

A MicroTek Model 220 gas chromatograph equipped with a ^{63}Ni electron-capture detector, and a Hewlett-Packard Model 5730A gas chromatograph with flame ionization detector were employed. Systems employed are listed below.

	Column Material	Inlet	Column	N_2flow	t_r
1.	3% OV-1 on Chromsorb WHP (100-120 mesh)	220	210	50 ml/min	4.4 min
2.	1.5% OV-17, 1.95% QF-1 on Chromosorb-W (80-100 mesh)	275	225	60 ml/min	12.8
3.	3% SE-30 on gas chrom Q (80-100 mesh)	275	235	60 ml/min	4.8
4.	10% UCW 932 on Chromosorb W (80-100 mesh)	250	225	50 ml/min	9.5

Columns were 180 cm in length.

The lower limit of detection in system 1 was 2 ng, and 10 ng in systems 2 and 3. At these lower levels, DCB appeared to decompose, with chromatograms showing several peaks in addition to DCB.

The utility of GLC in this study was limited by the presence of electron-capturing materials in pond and lake waters which interfered with DCB analysis.

Liquid Scintillation Counting

Radiometric determinations were made with a Model 3255 Packard Tri-Carb liquid scintillation spectrometer. Radioactivity in alkaline solutions containing $^{14}CO_2$ was determined using Instagel phosphor solution (Packard Instruments, Downers Grove, IL.). All other determinations were done with a phosphor solution containing 5.5 g 3,5-diphenyloxazole (PPO), 0.1 g 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP), 667 ml toluene, and 333 ml Triton X-100. Up to 1 ml of aqueous samples were mixed with 10 ml of the phosphor. Strongly acidic samples were neutralized prior to counting, to prevent quenching.

High Performance Liquid Chromatography

Analysis of DCB solutions by HPLC was performed with a Waters Associates (Milford, MA) liquid chromatograph (model M6000A) equipped with a uv detector (Schoeffel Instrument Corp., Westwood NJ, model GM770). A 4 mm (i.d.) X 30 cm

column packed with μ Bondapak C18 reversed phase medium (Waters Associates) was utilized. DCB was detected by its absorbtion at 282 nm.

Two solvent systems were utilized. The first, consisting of 1% (pH 5.5) ammonium phosphate buffer:acetonitrile (70:30 v/v) gave retention volumes for benzidine, monochlorobenzidine, and dichlorobenzidine of 6.9, 12.3, and 30 ml, respectively. One hundred nanograms or more of DCB gave a quantifiable response. The second system utilized was more suitable for routine analysis and consisted of acetonitrile:5% gl. acetic in water (70:30 v/v). Retention volumes for benzidine, monochlorobenzidine, and dichlorobenzidine were 6.8, 4.6, and 3.3 ml, respectively. Five nanograms of DCB were quantifiable in this system at maximum sensitivity. In addition, changing the relative proportion of acetonitrile and acetic acid affected the elution pattern seen. For example, with a 50:50 mixture of acetonitrile:acetic acid, the order of elution observed was the reverse of that from the 70:30 solvent mixture.

Prefiltration of samples prior to HPLC analysis is a recommended procedure to prevent clogging of the column by particulate matter in the samples. It was determined that up to 30% of DCB in distilled water solution was retained on 0.45 μ cellulose acetate filters (Millipore Corp., Bedford, MA) and over 60% on Fluoropore (Teflon over a polyethylene support) filters. In turbid lake water samples (from the microbial metabolism experiments), over 95% retention was noticed on both filter types. This problem was alleviated by addition of an equal volume of acetonitrile to samples prior to filtration with the Fluoropore filters. Filter retention of DCB from distilled and lake water was reduced to 1% or less (determined radiometrically) by this procedure. Samples containing ^{14}C-DCB were analyzed by scintillation counting before and after filtration to maintain a check on the efficiency of filtration. Subsequent studies revealed that the addition of acetonitrile also reversed the association of DCB with materials in lake water (Section 5), which probably caused the higher filter retention of DCB in lake water samples compared to distilled water samples.

HPLC proved to be the preferred method of analysis of DCB and analogs because it permitted direct determination in aqueous solution, thus eliminating a possible loss of the chemical through photodegradation during extraction and concentration steps required with other procedures. Also, the selectivity offered by the uv detector eliminated the need for sample clean-up prior to analysis.

EXPERIMENTAL PROCEDURES

Adsorption/Desorption of DCB to Aquatic Sediments

Bottom sediments from USDA pond, Hickory Hill pond, and Doe Run pond of the Athens, GA area were provided by the Athens Environmental Research Laboratory. The composition of these sediments is listed below.

Sediment Composition

Pond	pH	% Organic carbon	% Clay	% Silt	% Sand
USDA	6.4	0.8			
Doe Run	6.1	1.4	<1%	44%	56%
Hickory Hill	6.3	2.4	<1%	45%	55%

In addition, sediments from Lake Theriot, LA (an organic muck) and Eagle Lake, MS (a silt, reduced clay sediment) were obtained from the U.S. Army Corps of Engineers, Waterways Experiment Station, Vicksburg, MS. The sediments were passed through a screen to remove pebbles, leaves,and other detritus. After centrifugation to remove excess water, the sediment was thoroughly mixed. Moisture content was determined by drying portions of sediment at 90°C over dessicant until a constant weight was obtained.

To determine sorption, a solution of ^{14}C-DCB.2HCl was mixed with sediment to give a water:sediment (dry-weight equivalent) ratio of 100:1. The sediment suspension was contained in screw-cap centrifuge tubes with aluminum foil-lined caps and shaken on a wrist-action shaker, or in Erlenmeyer flasks shaken at 250 rpm on a rotary shaker. All samples were protected from light and maintained at 22°C. The shaking rate was adjusted to maintain the sediment in complete suspensions. At the appropriate time and at equilibrium, samples were centrifuged at 15000 rpm for 10 minutes, and ^{14}C content in the supernatant was determined by scintillation counting. The amount of ^{14}C-DCB disappearing from the solution was assumed to be sorbed by the sediment. Adsorption of DCB to glass was not detected over 24 hours in centrifuge tubes or Erlenmeyer flasks containing a solution of ^{14}C-DCB in distilled water. The effect of pH on sorption was studied in appropriately buffered solutions: pH5 (0.01M acetate buffer), pH7 (0.01M phosphate buffer), pH 9 (0.01M borate buffer). All other sorption studies were done in distilled water-sediment suspensions. Except where otherwise noted, sorption solutions contained an initial DCB.2HCl concentration of 2.0 ppm.

The effect of DCB.2HCl concentration on the degree of sorption was measured for the Hickory Hill pond and Lake Theriot sediments. Sediments were suspended in solutions containing 0.5 - 2.0 ppm DCB and sorption determined as described above.

Desorption was studied in sediments that were suspended in 2 ppm DCB.2HCl for either 24 hours or 7 days, at which time sorption was measured and desorption initiated. After centrifugation, the supernatant solution was removed, and its ^{14}C content determined. The sediment pellet was washed once by suspension in distilled H_2O, to remove DCB from the solution that may have been trapped in interstitial spaces of the pellet. After ^{14}C determination, the wash was discarded, an appropriate volume of distilled water was added (maintained a liquid/sediment ratio of 100), a 0 time sample was taken,

and the samples were then shaken vigorously for up to 72 hours. During this period, the suspension was centrifuged at appropriate intervals and the supernatant was counted for ^{14}C to determine the amount of desorbed DCB. Extraction of DCB from sediment by organic solvents and other agents was performed by similar procedures.

Photodegradation

Studies on the photodegradation of DCB were carried out in a preparative photochemical reactor (Ace Glass Company) system. The reaction system consists of a jacketed borosilicate glass vessel and is equipped with a side arm for withdrawing samples. A double-walled water-cooled quartz well, housing the light source and filter, is fitted into the vessel and is immersed in the solution to be irradiated. The aqueous solution of DCB was irradiated with a 400 watt Hanovia medium pressure mercury lamp fitted with a Pyrex 7740 filter to exclude light of wavelength less than 300 nm. Aliquots of the photolyzed solution were withdrawn at appropriate intervals and analyzed for DCB and its degradation products. Quantum yield was measured using a Rayonet "merry-go-round" apparatus.

Analysis of Photoproducts --

Photolyzed aliquots were basified and extracted with ether, and the dried ($MgSO_4$) ether concentrate was spotted on a silica-gel TLC plate and developed in ether:hexane (2:1). A spot which co-chromatographed with benzidine was scanned with a spectrofluorimeter, and its spectrum was found to be identical to that of pure benzidine. The presence of benzidine was further confirmed by comparison of its GC (20 in. UCW 982 on Chromosorb W and OV-1) and HPLC retention times with an authentic sample and by GC-MS analysis. MCB was identified by GC-MS and by comparison of GC and HPLC retention times with an authentic sample (synthesized from nitrobenzene and o-chloro-aniline according to Branch et al. (5).

Photodegradation of DCB in Natural Sunlight --

To determine the rate of photodegradation of DCB in natural sunlight, a 3.6 ppm aqueous solution of DCB.2HCl was divided into eight 5 ml portions in tightly stoppered quartz tubes that were exposed to sunlight on a clear day (Aug. 24, 1977, 10 A.M. EST) on the roof of our laboratory. The tubes were covered with aluminum foil every 30 secs. until a maximum exposure time of 4 minutes was reached.

Degradation of DCB by Aquatic Microorganisms

These studies were done using samples of lake water and sediment and in activated sludge.

Microbial Degradation in Lake Water and Sediment --

Samples of water and sediment were obtained from the following sources: (i) Oneida Lake - a large mesotrophic lake approximately 20 miles northeast of Syracuse, NY, (ii) Jamesville Reservoir - a small eutrophic lake about

10 miles southeast of Syracuse. Microbial degradation of DCB was examined in water alone and water + sediment. Prior to starting the biodegradation studies, microbial counts in the water samples were made using the standard serial dilution and plating techniques (6).

To determine the rate of biodegradation of DCB, non-radioactive DCB was added to the water in Erlenmeyer flasks at a concentration of 2 ppm. The flasks were stoppered with foam plugs and incubated on a gyrotary shaker in the dark at 21 $\pm$ 1°C. Sterilized water samples containing DCB were included as controls to account for any non-biological degradation. At appropriate intervals, the contents of the flasks were analyzed for DCB by HPLC as described previously. The rate of degradation of DCB in the water was also examined in the presence of sediment. DCB was added to flasks containing 50 ml water and 0.5 g lake sediment (dry wt. equivalent). At suitable intervals, samples of the aqueous solution above the sediment were removed and centrifuged to remove suspended material and the concentration of DCB in the water was determined. The sediment was shaken with methanol to remove adsorbed material for analysis.

Carbon-14-labeled DCB was used to determine the products resulting from the biodegradation of the chemical. Carbon-14-DCB was added to the water samples at a concentration of 2 ppm. At periodic intervals, the water samples were adjusted to pH 11, shaken with ether, and the ^{14}C distribution between the aqueous and organic phases was determined. The ether extracts were analyzed by TLC. Additional search for metabolic products was performed by HPLC coupled with scintillation counting of the eluted fractions.

The evolution of $^{14}CO_2$ was measured using the biometer flask described by Bartha and Pramer (7). The water samples were incubated with 2 ppm of ^{14}C-DCB in the biometer flask and 0.1N KOH was used as the CO_2-trapping solution in the side arm. The solution in the side arm was removed and replaced at appropriate intervals. The amount of ^{14}C in the CO_2-trapping solution was measured by liquid scintillation counting. The radioactivity collected in the KOH trap was verified as $^{14}CO_2$ by acidifying with HCl.

Microbial Degradation in Activated Sludge --

Activated sludge for this study was obtained from a local sewage treatment plant. The sludge was adjusted to a MLSS concentration of 1000 mg/l. Carbon-14-DCB was added to the sludge at a concentration of 2 ppm. The flasks containing the sludge and DCB were incubated on a rotary shaker at 21°C $\pm$ 1°. Periodically, aliquots were removed, diluted with acetonitrile, and centrifuged. The precipitated solid sludge was also extracted with methanol, and both the water and methanol extracts analyzed by HPLC.

To minimize the loss of the chemical because of adsorption on the sludge solids and to determine whether the microorganisms in activated sludge would adapt to degrading DCB, a separate experiment on the biodegradation of DCB was conducted using activated sludge as a microbial inoculum (8). In this test, 10 ml of settled activated-sludge supernatant was added as a source of microbial inoculum to 90 ml of a mineral medium (8) containing 50 or 100 mg of yeast extract and 2 ppm of DCB. After an incubation period of 7 days,

10 ml of the contents from the inoculated flask were withdrawn and transferred to a fresh medium containing the DCB and the procedure was repeated for three consecutive weeks. After incubation for 7 days, each sub-culture was analyzed for DCB.

Release of Free DCB from DCB-based Pigments --

Carbon-14-Yellow Pigment 12 was prepared by the coupling of ^{14}C-(UL)-DCB.2HCl with two equivalents of acetoacetanilide by a diazotization procedure provided by the chemists of Sun Chemical Corp., Staten Island, NY. The purity of the product was not determined because of the insolubility of the pigment in water or organic solvent. The product was repeatedly washed with methanol and no ^{14}C-DCB was detected in the final wash.

Carbon-14-Yellow Pigment 12 was mixed with test water (added as a concentrated acetone suspension) to give a theoretical pigment concentration of 4 ppm. The pigment was incubated with the following types of water: (i) sterilized distilled water, (ii) Muskegon County, Michigan Waste Treatment Lagoon water (this lagoon receives the effluent of a DCB-utilizing factory), (iii) Jamesville Reservoir water, and (iv) Oneida Lake water. The samples were shaken in the dark at a temperature of 21 $\pm$ 1°C.

Aliquots of water were sampled weekly, adjusted to pH 10, and shaken with ether. Because of the low quantity of ^{14}C detected in these extracts, further analysis was not done until after 28 days of incubation. At this time, the entire water sample was sacrificed, pH adjusted to 10, and shaken with ether. The extracts were fortified with non-radioactive DCB, divided into two portions, and concentrated. One portion was applied to thin-layer chromatographic plates and developed in a system of ether:hexane (3:1 v/v) along with a known DCB spot. The other portion was developed in benzene: ethyl acetate:acetic acid (7:3:0.1). After development, the DCB-containing area of the plates was located under a uv lamp and removed from the plate. The ^{14}C was eluted from the silica gel with methanol, and determined by scintillation counting. Methanol eluted 84% of authentic ^{14}C-DCB from silica gel, which was considered in calculating recovery of DCB in authentic samples.

Uptake, Elimination and Metabolism of DCB in Bluegills

Bluegills (Lepomis macrochirus Raf.) (2 to 3 in. in length) were obtained from the National Fish Hatchery, Orangeburg, SC, and from a commercial hatchery. Fish were acclimatized to laboratory conditions for 30 days under a 14 hr light/10 hr dark cycle and fed a pelleted diet.

Exposures were conducted at 21°C in all-glass containers protected from light. Solutions were mildly aerated during exposures. A preliminary experiment showed no loss of DCB over 7 days under these conditions, in the absence of fish. Because this solution was prepared from water taken from fish-holding aquaria, contribution to DCB metabolism under test conditions by fish associated microbes appears negligible.

The fish were removed from the treated water at appropriate intervals, rinsed with clean water, and sacrificed. The content and distribution of ^{14}C-DCB and derived materials in exposed fish was measured in the following manner. After sacrifice, the fish were dissected into two portions prior to analysis: the head plus viscera fraction (consisting of the head, gills, and internal organs), and the edible flesh fraction (flesh and skin). After weighing, the tissue fractions were homogenized with methanol (5 ml/g fresh weight) in a blender. The slurry was centrifuged and the supernatant was decanted. The residue was reextracted with methanol. After centrifugation, the two extracts were combined and the amount of ^{14}C in the pooled extract was determined by liquid scintillation counting. The amount of ^{14}C in the tissue residue was determined by solubilizing it in the NCS tissue solubilizer (Amersham Searle Corp.) for 48 hours at 50°C, as described by Sikka et al. (9). Glacial acetic acid (0.02 ml/ml of solubilizer) was added to the solubilized tissue and the solution was counted for ^{14}C using scintillation fluid containing Triton X-100. The samples were stored overnight at 4°C in the dark before counting. The radioactivity in the methanol extract and in the tissue residue was combined to calculate the ^{14}C concentration in the fish. The whole fish ^{14}C residues were calculated from the combined values of the edible flesh and head plus viscera fractions. To study the metabolism of DCB by the fish, the methanol extracts of the tissues were analyzed by partition extraction, TLC and HPLC (Figure 5, Section 5).

The nature of the ^{14}C remaining in the water bathing the fish was also determined. After removing the fish, the water was adjusted to pH 10 and extracted with diethyl ether. The amount of ^{14}C in the organic and aqueous phases was determined. The ether extract was concentrated and aliquots were chromatographed on thin-layer silica gel plates.

SECTION 5

RESULTS AND DISCUSSION

ADSORPTION AND DESORPTION OF DCB IN AQUATIC SEDIMENTS

Results

The rate of DCB adsorption to all sediments studied was initially very rapid (Figure 1). The end-point of the sorption was generally achieved within the first 24 hours of the experiments. The comparative distribution coefficients (K_d = 100 x ug DCB sorbed/ug DCB in solution) listed in Table 1 indicate the high affinity of DCB for a variety of sediments. In addition, sorption was reduced significantly at pH 9, compared to values obtained at pH values of 5 or 7 (Table 2).

TABLE 1. DISTRIBUTION COEFFICIENTS (K_d) OF SEDIMENT SORPTION OF DCB

Sediment	K_d *
USDA Pond	2850 (666)
Hickory Hill Pond	3210 (328)
Doe Run Pond	2670 (310)
Lake Theriot	8866 (166)
Eagle Lake	12800 (2776)

*Values are the average of three replications. Figures in parentheses are standard deviations.

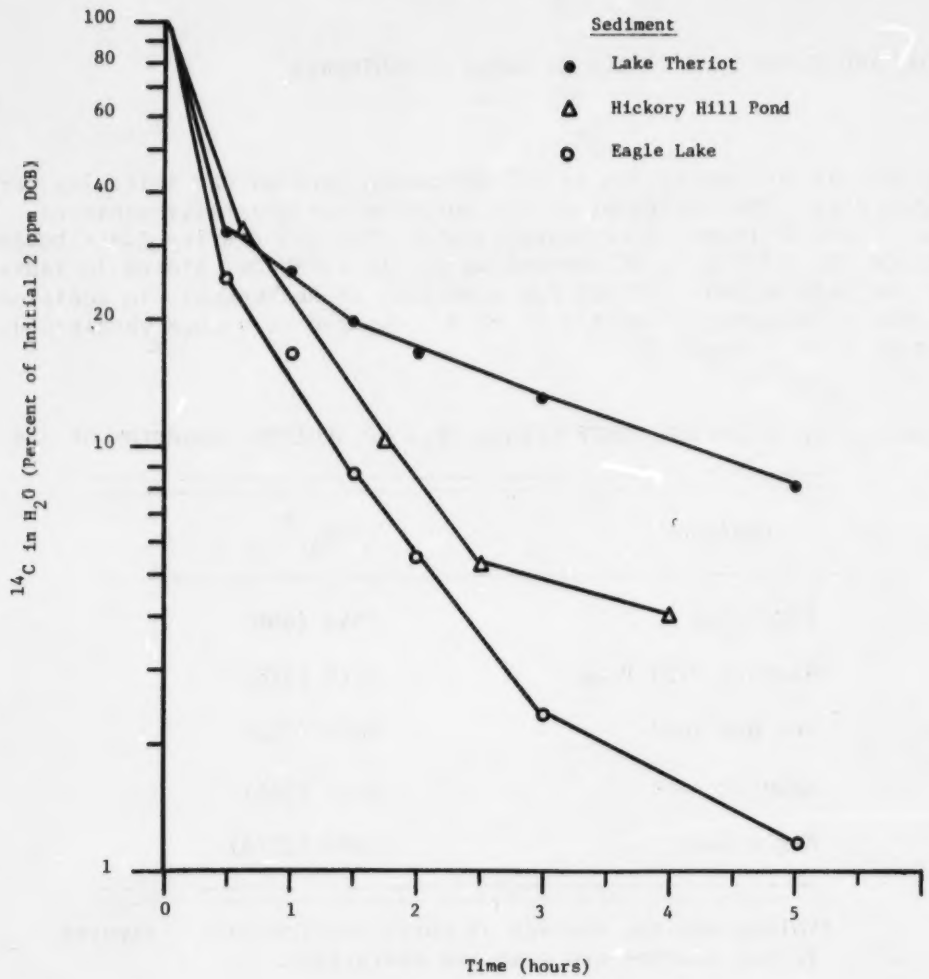

Figure 1. Rate of adsorption of DCB by aquatic sediments (1g sediment per 100 ml solution).

TABLE 2. EFFECT OF pH ON SORPTION BY DCB BY SEDIMENT

Sediment	K_d* pH5	pH7	pH9
Doe Run Pond	3325 (655)	2417 (295)	206 (18)
Hickory Hill Pond	2225 (252)	2122 (545)	269 (98)
USDA Pond	3783 (1254)	3083 (445)	95 (2)

*The values are the average of three replications. Values in parentheses are standard deviation.

Equilibrium adsorption isotherms for Hickory Hill pond and Lake Theriot sediments are presented in Figures 2 and 3. Using the equation of Freundlich,

$$x/m = kc_e^{1/n}$$

where x = ug adsorbed DCB, m = dry weight of sediment, and c_e = equilibrium (or end-point) concentration of DCB in solution, the constant 1/n was obtained from the slope of the plot of log x/m against log c_e and was found to be near unity. The constant k was obtained from the slope of a plot of x/m against c_e. Slopes were obtained by a least squares method, with correlation coefficients exceeding 0.98. These values are given in Table 3. The experimental K_d values obtained with 2 ppm DCB.2HCl in separate studies approximate the k values obtained by this approach, although the K_d for the Hickory Hill sediment is somewhat lower.

Investigation of the desorption of DCB confirmed the high affinity for sediment. Preliminary studies showed that DCB desorption was related to the age of the sample at the time when desorption was initiated. That is, a higher degree of desorption was obtained from samples in which DCB had been in contact with sediment for one day than with those where contact had been maintained for several days. Therefore, further desorption studies included two samples types: sediment suspended in tCB solutions for either 24 hours or 7 days.

TABLE 3. SORPTION OF DCB BY SEDIMENT*

Sediment	1/n	k
Hickory Hill	0.96	4,984
Lake Theriot	0.79	5,952

*Values were obtained from triplicate sorption determinations at each test concentration by regression analysis ($r > 0.98$).

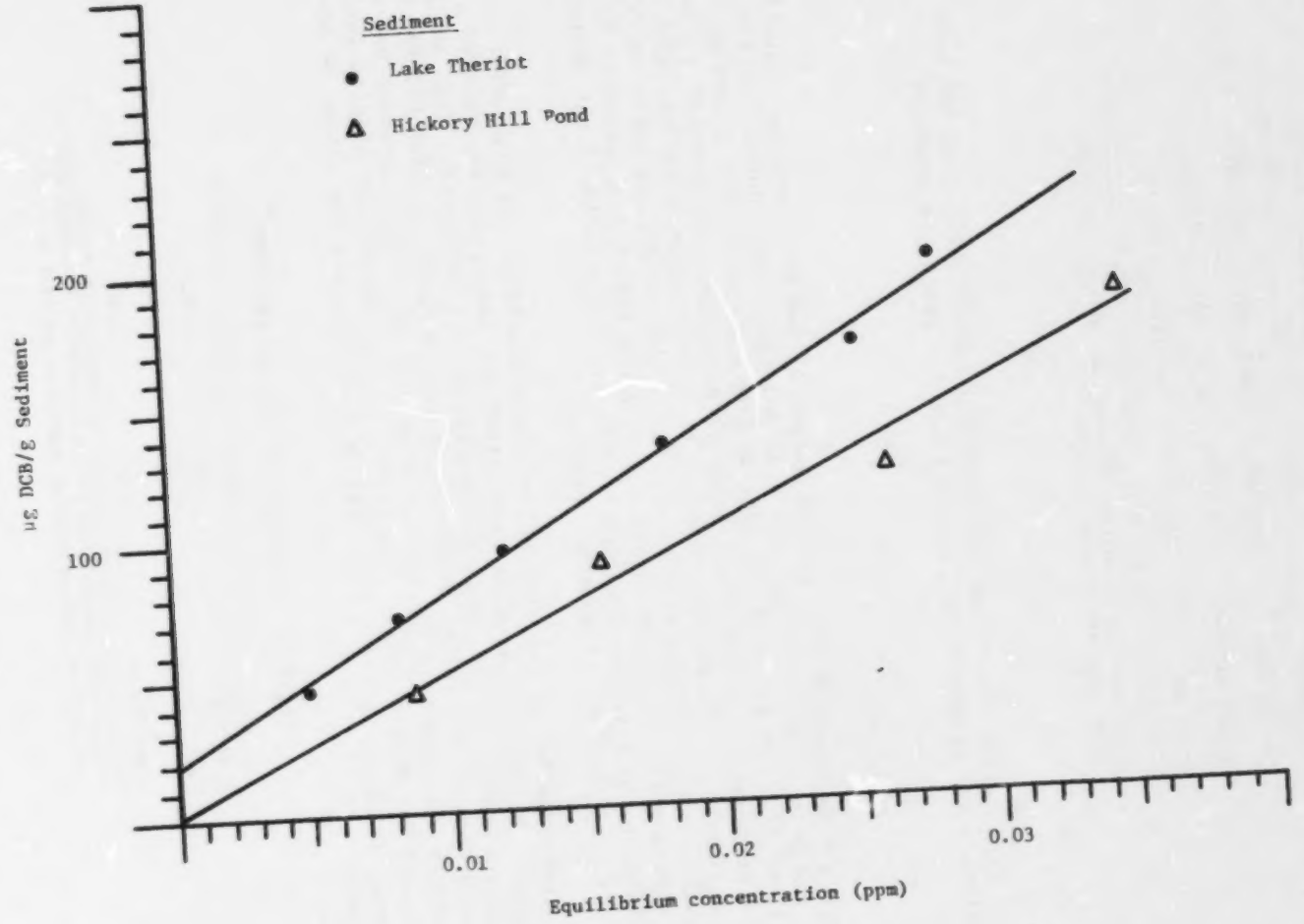

Figure 2. DCB adsorption as a function of DCB concentration.

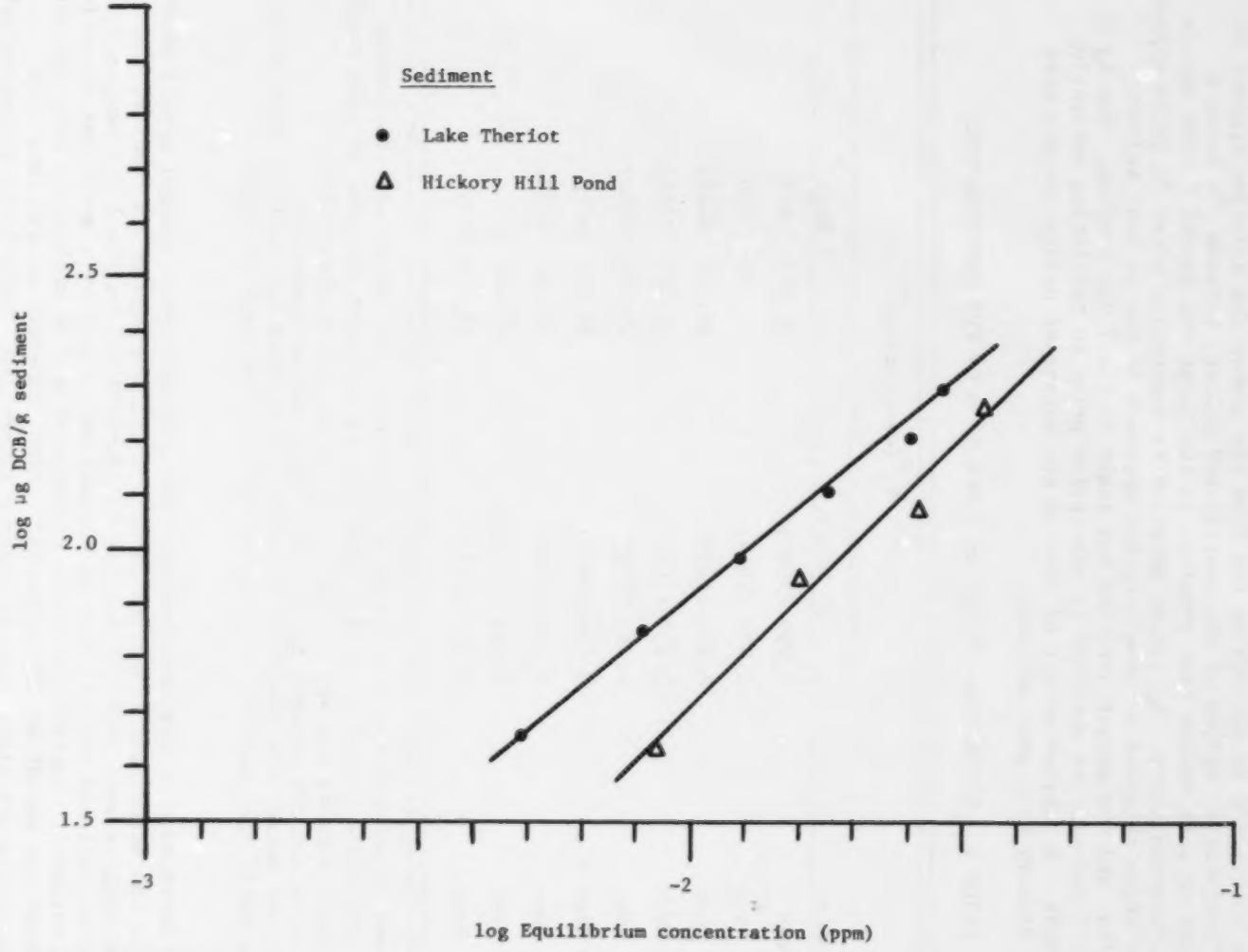

Figure 3. DCB adsorption isotherms.

The desorption of DCB from Lake Theriot sediment is listed in Table 4. In the 24 hour samples, desorption rapidly approached an apparent equilibrium as indicated by ^{14}C recovered in the washes and 0 times of the desorption phase. This ^{14}C is not due to DCB from the adsorption solution trapped in the interstitial spaces of the centrifuged pellet, because ^{14}C amounts similar to wash values were obtained in the first and second 0 time desorption determinations. K_d values obtained by desorption after 72 hours approximate those obtained by an adsorption approach in the 24 hour sediment samples, and the amount desorbed was lower in the 7 day samples. The K_d of these 7 day samples obtained by adsorption prior to initiating desorption was 6354. A similar effect of time on the extent of desorption was seen with Hickory Hill pond sediment.

TABLE 4. DESORPTION OF DCB AS A FUNCTION OF PRE-EXPOSURE TIME

	K_d (desorption)*	
Sample	24 Hours †	7 Days ‡
Wash	9,999 (2011)	32,053 (1843)
0 Hour	12,773 (1914)	32,276 (3830)
3 Hour	6,896 (1209)	36,113 (8412)
24 Hour	8,262 (206)	25,322 (5742)
48 Hour	6,837 (778)	16,903 (1164)
0 Hour #	10,726 (1832)	51,014 (6474)
24 Hour	8,101 (339)	41,393 (6439)
48 Hour	6,814 (371)	15,462 (173)
72 Hour	6,974 (319)	14,228 (93)

*Values in parentheses are standard deviation. The values are the average of triplicate determinations. Lake Theriot sediment was used in this study.
†Sediment samples exposed to DCB for 24 hours before desorption.
‡Sediment samples exposed to DCB for 7 days before desorption.
#After 48 hours, the desorbant solution was removed and replaced with fresh desorbant; and desorption monitored for an additional 72 hours.

Extraction of DCB from sediment was attempted with several agents at the end of the adsorption phase (Table 5). As in the desorption studies, the 7-day samples were more intractable to extraction than the 1-day samples. Other extractants tested, such as toluene and ether, were much less effective than methanol. Extraction of the sediment with 0.5N methanolic NaOH did not increase the amount of DCB recovered. Also, repeated extractions with solvents did not significantly improve recoveries. Thin-layer chromatography of the methanol extracts showed only DCB.

TABLE 5. EXTRACTION OF DCB FROM SEDIMENT

Agent	Percent ^{14}C extracted*	
	Period of sediment exposure to ^{14}C-DCB	
	24 Hours	7 Days
5N NH_4Cl	2.1	0.7
1N HCl †	9.0	3.1
1N NaOH	31.3	16.7
Methanol	36.2#	21.9

*Values are the average of three replicates.
†HCl extraction data were obtained from Lake Theriot sediment; all other data were obtained from Hickory Hill sediment.
#Extraction of these samples with a second portion of methanol liberated an additional 3.8% of adsorbed ^{14}C.

Discussion

No obvious relationship between organic carbon content of the sediments and the degree of sorption was observed, even though organic matter is believed to be the major site of sorption of most hydrophobic chemicals (10). Given that the distribution equilibria so heavily favor sorption, it may be that even sediments relatively deficient in organic matter possess sufficient adsorbant to favor nearly complete DCB sorption under the experimental conditions. In preliminary experiments, sediment concentrations of DCB of 700 ppm or more could be achieved by repeated exposure of sediment to 2 ppm DCB solutions or by suspending 1 g of sediment in 500 ml of a 2 ppm DCB solution.

The linear isotherms obtained from the sediments studied show that DCB sorption may be described by the mathematical treatment of Freundlich, within the concentration limits of this study.

Studies have shown that desorption of lipophilic substances such as toxaphene (11) from sediment in aqueous solution is extremely slow or insignificant. This condition was seen in the 7-day DCB-sediment samples, although the 24 hour sediments studied rapidly established a DCB distribution with the water similar to that obtained by an adsorption approach. Therefore, extrapolation on the role of absorption and, especially, desorption in determining the long-term environmental fate of DCB, should be made with caution until further work establishes the time-dependent nature of the DCB-sediment complex.

The inability of 5N NH_4Cl or 1N $CaCl_2$ to appreciably enhance desorption of DCB from the sediments studied would indicate the cation-exchange

mechanisms may play a minimal role, if any, in sorption of DCB under normal environmental conditions (12). Because the pK_a of DCB is less than 4, DCB is predominantly in a neutral state under our experimental conditions, thus minimizing any potential ionic interactions between DCB and sediment. For the same reason, it is unlikely that the decrease in sorption seen under alkaline conditions in this study is caused by repression of ionic mechanisms. As another possible contributing factor, alkaline conditions may change the sorptive properties of the sediments. For instance, it is known that treatment of sediments with NaOH releases humic and fulvic acids from soil or sediment (13). In this light, it is interesting to note that sorption at pH9 was reduced by approximately 30-50% (Table 2), and that treatment of DCB-sediment with 1N NaOH released over 30% of sorbed ^{14}C from the sediment (Table 5), indicating the possibility that alkaline conditions may solubilize or otherwise modify a portion of the sediment components responsible for adsorption of DCB.

In this study, a high degree of difficulty was encountered in extracting more than a fraction of adsorbed DCB from sediment. Also, the efficiency of desorption and extraction decreased according to the length of time DCB had been in contact with the sediment. These observations are consistent with the occurrence of chemical reactions between DCB and sediment constituents. This process has been noted with other chemicals, including aromatic amines. For example, Hsu and Bartha (14,15) have demonstrated that chloroanilines react with organic constituents of soil, forming covalent complexes, by condensation of the amine moiety with carbonyl and other functions in the soil. Portions of these complexes are resistant to acid or base hydrolysis, and the amount of the complex increases with time. This mechanism may apply to other amines as well. For instance, it was recently shown that aminoparathion is very highly bound to soils, a condition not seen with the parent chemical, parathion (16). It is likely that this process would lead to formation of humic-like materials that presumably would be non-hazardous (13). These findings for chemicals that are structurally similar to DCB are consistent with our observations. Although there is little doubt that the rapid decrease in free DCB from aqueous suspensions of sediment is predominantly the result of a phycial adsorptive process; it seems likely that, once adsorbed, DCB may covalently bind to the sediment and become increasingly resistant to desorption and extraction as this process occurs over time.

PHOTODEGRADATION OF DCB

Results

Products of DCB Photodegradation --

In our preliminary studies, aqueous solutions of DCB.2HCl (approx. 5×10^{-5}M) were photolyzed for periods ranging from 5-30 min with a 450W medium pressure mercury lamp equipped with a Pyrex filter. A red-brown material was found to deposit on the outside surface of the well. The uv spectrum of the resulting solution showed a decrease in absorbance with respect to the original solution as well as a slight hypsochromic shift.

Analysis of the solution showed that it contained benzidine. The red material that deposited on the walls of the photoreactor was dissolved in ether and the ether concentrate was worked up and spotted on a silica gel TLC plate and developed with ether:hexane (2:1). Five to eight brightly colored bands were observed.

Finally, a solution of DCB in hexane was photolyzed for 30 min, and concentrated. TLC analysis showed only one spot corresponding to DCB, and no degradation products were observed.

To determine the time course of DCB photodegradation and the appearance of products, an aqueous solution of DCB.2HCl was photolyzed for 45 minutes. Aliquots withdrawn at periodic intervals were analyzed for DCB, MCB, and benzidine by HPLC. The results presented in Table 6 clearly demonstrate that DCB is readily degraded by light; more than half of the chemical had disappeared within four minutes of exposure. On the basis of these findings, we conclude that DCB (in part) is photolyzed sequentially to MCB and benzidine.

TABLE 6. PHOTOLYSIS OF DCB.2HCl

Minutes of irradiation	DCB.2HCl* x 10^7M	MCB.2HCl* x 10^7M	Benzidine.2HCl* x 10^7M
0	20	--	--
1	18	1.2	--
2	17	2.1	--
3	13	2.3	--
4	7.4	3.9	--
5	2.4	1.5	1.7
10	--	1.5	2.8
15	--	--	3.3
45	--	1.2	2.8

*Average of two determinations.

In order to have some information on the mass balance of the products resulting from the photodegradation of DCB, preliminary studies using ^{14}C-DCB.2HCl were carried out. An aqueous solution of ^{14}C-DCB.2HCl was irradiated for an hour, and samples were periodically withdrawn, basified and extracted with benzene. The ^{14}C measured in the benzene and aqueous fractions are listed in Table 7. It is evident that a significant portion of substrate is converted to acidic materials which have a high affinity for the aqueous phase. Furthermore, a large fraction of the original material was converted to insoluble products that were retained on the walls of the

photoreactor. This was confirmed in a subsequent experiment in which an aqueous solution of ^{14}C-labeled DCB.2HCl was irradiated for one hour. As before, the resulting solution was found to contain less radioactivity than the starting solution. The photoreactor was rinsed with ether, and the missing ^{14}C was found to be in the ethereal solution.

TABLE 7. PHOTODEGRADATION OF ^{14}C-LABELED DCB

Time (minutes)	Percent of initial ^{14}C in benzene phase	Percent of initial ^{14}C in aqueous phase *
2	97.4	3.08
5	80.9	12.4
7	54.6	21.2
12	40.2	16.4
15	30.4	13.9
30	23.4	16.5
60	12.4	12.2
135	5.6	16.4
150	4.6	22.2

*After extraction with benzene.

Photodegradation of DCB in Natural Sunlight --

To determine the rate of photodegradation of DCB in natural sunlight, a 3.6 ppm aqueous solution of DCB.2HCl was photolyzed for 4 minutes. The DCB and MCB levels were measured by HPLC and are presented in Table 8. No benzidine could be detected. However, in an earlier preliminary experiment in which the solution was exposed to sunlight for 19 minutes to 5 hours, the presence of benzidine was observed.

Lifetime of Transients Generated from the Photolysis of Chlorobenzidines --

During our experiments on the photodegradation of dichlorobenzidine, we noticed the appearance of a green color that disappeared when the photolyzed solution was allowed to stand in the dark. In an attempt to understand the mechanism involving the disappearance of the green intermediates, an experiment was conducted in which DCB and MCB were irradiated with 2537A light in a Rayonet "merry-go-round" photoreactor.

Irradiation of aqueous solutions of DCB and MCB with a 2.2W mercury arc at 2537Å (Rayonet minireactor) for approximately one minute led to the formation of green intermediates which decayed in the dark. The rate of decay, which appeared to be slower in acidic solutions, was monitored spectrophotometrically at the absorption maximum of the transient and was of the first

order with respect to substrate (correlation coefficient of the linear regression line >0.999). The results are presented in Table 9.

TABLE 8. PHOTODEGRADATION OF DCB IN NATURAL SUNLIGHT

Time (seconds)	DCB.2HCl* x 10^6M	MCB.2HCl* x 10^6M
0	11	0
30	9.8	1.5
60	6.7	2.4
90	5.7	2.3
120	4.8	3.2
150	4.4	3.7
180	3.3	3.6
210	2.6	3.4

*Average of two determinations.

It is difficult to interpret these results in an absence of a knowledge of the structure of the transients. It is evident, however, that the rate is dependent upon initial substrate concentration to a limited extent, and more importantly that the similarities of the rates for DCB and MCB point to a common mechanism. In other experiments conducted in solutions of higher pH, the rate of disappearance of the transients increased markedly.

Measurement of Quantum Yields

The disappearance quantum yields of benzidine and MCB were measured in a merry-go-round apparatus with respect to a ferrioxalate actinometer at 2537Å and 3000Å. The wavelength dependence of the quantum yields was minimal, and the results obtained at 3000Å are reported in Table 10. The limited solubility of DCB in water made direct determination of the quantum yield at 3000Å difficult, and consequently the measurement was made with respect to MCB at 2537Å. The high quantum yields for DCB and MCB confirm the photolability of these substrates whereas the relatively low value for benzidine is in keeping with Metcalf's finding that the disappearance half-life of benzidine in methanol at 254 nm. is about 2 hours (17).

A number of experiments were also conducted to determine the pH dependence of the quantum yield for DCB. These results, presented in Table 11, indicate that the rate of degradation is acid catalyzed. The appearance quantum yields for MCB, although less accurate due to the reactivity of this material, also appear to be acid catalyzed. In a future, more mechanistically oriented study, we will determine the rate profile for the conversion of MCB to the relatively more stable benzidine.

Photolysis in Organic Solvents --

To determine the photoreactivity of DCB in solvents other than water, a number of preliminary experiments were carried out in hexane, isopropanol and methanol. Irradiation of these solutions under approximately the same conditions under which disappearance of DCB in aqueous solution is virtually complete showed that very little degradation had occurred. For example, irradiation of a 15 ppm solution of DCB.2HCl in iso-propanol at 2537Å for one hour led to 28% degradation. Under similar conditions, a 4 ppm aqueous solution of DCB is decomposed to an extent of 42% in 0.3 minutes. It is therefore evident that photodegradation of DCB in water proceeds through a mechanism different from that in organic solvents.

Mechanistic Considerations --

Recent studies on aromatic dehalogenation in aprotic solvents has shown that the mechanism involves homolysis of the carbon-chlorine bond, followed by hydrogen abstraction from solvent (18-20). In aqueous media, irradiation of chloroaromatics frequently leads to photosubstituted products, presumably through the nucleophilic photosubstitution mechanism advocated by Havinga and coworkers (21). While most of the photochemistry of chloroaromatics may be rationalized in terms of these two mechanisms, examples do exist where other pathways must be considered. Nordblum and Miller (22) observed that irradiation of 4,4'-dichloro-biphenyl in ether containing 2% trifluoroacetic acid gave 4-chloro-biphenyl. In hexane, cyclohexane, acetonitrile or pure ether, complex product mixtures were obtained. Furthermore, the reduction is accompanied by a kinetic isotope effect, as determined by the use of deuterated trifluoroacetic acid. Simple bond homolysis does not explain the acid catalysis or the large isotope effect.

The photoreduction of DCB would also appear to involve a mechanism different from simple bond homolysis. The relative inertness of the material in isopropanol or hexane, both of these solvents being appreciably better hydrogen atom donors than water, implies the specific involvement of water in the transition state. While the limited scope of our work does not allow further mechanistic speculation at present, it appears that proton transfer is involved. We are presently investigating the mechanism of the reaction in somewhat greater detail.

Conclusions

DCB is very rapidly photodegraded under enviornmental conditions through reductive dechlorination and other processes. In organic solvents, the reaction is considerably slower. The mechanism of dechlorination while as yet unknown, nevertheless does not involve carbon-chlorine bond homolysis.

TABLE 9. KINETICS OF DISAPPEARANCE OF TRANSIENTS GENERATED FROM THE PHOTOLYSIS OF DCB AND MCB

[DCB.2HCl] x 10^{5}*	HCl(N)	λ(nm.)	T(°C)	k(min^{-1}) x 10^{2}
2.15	1.00	425	22.5	8.50
2.15	1.00	"	22.2	8.87
2.15	1.00	"	"	7.61
44.5	0.12	"	"	12.8
44.5	0.12	"	"	13.1
5.61	1.00	"	"	12.8
11.2	1.00	"	"	15.8
16.8	1.00	"	"	16.5
22.4	1.00	"	"	18.9
28.0	1.00	"	"	14.1
[MCB.2HCl] x 10^{4}*				
7.63†	1.00	435	22.5	19.1
"	1.00	"	"	18.6
"	1.00	"	"	18.3
"	1.00	"	"	18.6
7.63‡	1.00	435	22.5	18.3
"	1.00	"	"	16.8

* Initial concentration (molar).
† Photolyzed for 1 minute.
‡ Photolyzed for 2 minutes.

TABLE 10. DISAPPEARANCE QUANTUM YIELDS FOR DCB, MCB AND BENZIDINE

	ϕ	λ (Å)	pH
Benzidine	0.012	2537, 3000	7.0, 8.2
MCB	0.70	2537, 3000	7.0, 8.2
DCB	0.43	2537	6.7, 8.1

TABLE 11. pH DEPENDENCE OF THE RATE OF DISAPPEARANCE OF DCB

pH	$\phi_{rel.}$ (DCB)	$\phi_{rel.}$ (MCB)
1.96	1.0	1.0
3.96	0.71	0.73
6.01	0.42	0.49
8.28	0.50	0.56

BIODEGRADATION OF DCB BY AQUATIC MICROORGANISMS

Results

Microbial Degradation in Lake Water and Sediment --

A preliminary investigation utilizing Oneida Lake and Jamesville Reservoir water showed a negligible loss of DCB over a 3-week period. This study was initiated during May, 1977, when microbial populations were relatively low (less than 500 cells/ml). In order to assess the degradation of DCB under conditions of greater microbial activity, an additional study was initiated in mid-July when microbial assay of Jamesville Reservoir water showed approximately 5 x 10^6 cells/ml and Oneida Lake water 900 cells/ml.

As shown in Table 12 (first experiment), the DCB assayed decreased with time in both lake water sources. No degradation products were detected by either HPLC or TLC of the samples, and ^{14}C trapped in biometer flasks was less than 0.1% over 14 days in both lake water and sterile samples.

During the course of incubation, the samples became increasingly turbid, probably as a result of microbial growth and the accumulation of metabolic by-products (lipid, protein) and dead microbes. This accumulated material might have provided a surface for DCB adsorption. However, the specific activity of ^{14}C-DCB in this experiment was too low to provide an accurate determination of the amount of ^{14}C in the organic matter itself. Therefore, Jamesville Reservoir water (5 x 10^6 cells/ml) was incubated with ^{14}C-DCB.2HCl of higher specific activity to facilitate determination of the distribution of ^{14}C in the incubation medium. As in the previous study, a progressive loss of DCB was observed by HPLC over time (Table 12, second experiment), but no degradation products were detected. Also, as before, sample turbidity increased over time.

Portions of the 30-day samples prior to acetronitrile treatment were centrifuged, with 64.4% of original ^{14}C detected in the supernatant. In contrast, the distribution seen in acetonitrile-treated samples was 94.7% in the supernatant and 3.3% in the precipitate. Because there was a discrepancy between the amount of ^{14}C-labelled material equivalent to DCB in the

TABLE 12. PERSISTENCE OF DCB IN LAKE WATER

Time (days)	DCB concentration (ppm)*	
First Experiment	Jamesville Reservoir	Oneida Lake
0	2.07	2.26
7	1.81	2.22
21	1.55	1.66
28	1.47	1.68
Second Experiment	Sterile Control	Jamesville Reservoir
0	2.07	1.96
10	2.02	1.68
20	2.03	1.56
30	2.04	1.52

*As analyzed by high performance liquid chromatography. Values are the average of three replicates.

supernatant acetonitrile diluted samples (1.86 ppm) and the amount of DCB assayed in the samples by HPLC (1.52 ppm), portions of the 30-day samples containing known amounts of ^{14}C were applied to the HPLC column, eluted fractions collected, and the ^{14}C content of the fractions determined. The eluted fraction containing DCB showed 85.6 $\pm$ 3.8% of the applied radioactivity. Carbon-14 in the fractions before and after DCB, although present, was too low to quantify. It appears, therefore, that a portion of ^{14}C-material in the lake water samples does not chromatograph distinctly with the HPLC systems employed. In addition, portions of the 30-day samples were treated with acetonitrile and centrifuged. The supernatant was then basified and partitioned with ether. Only 0.9% of the original supernatant ^{14}C remained in the aqueous phase after ether partition. The extracts were analyzed by TLC. No distinct materials other than DCB were seen, under conditions capable of detecting components that represented 5% or more of the material applied to the plate. It seems likely, therefore, that the ^{14}C-material unaccountable by HPLC as DCB is indistinguishable by TLC as well.

After 4 weeks, samples containing water + sediment showed only 0.7% (Oneida Lake) to 3.6% (Jamesville Reservoir) of the original ^{14}C in the water after centrifugation. Values were 1.3% (Oneida Lake) and 2.0% (Jamesville Reservoir) for sterile control samples. Exhaustive extraction with methanol removed only 29 to 42% of the original ^{14}C from the sediment. The extracted ^{14}C was shown to be DCB by TLC. A further experiment in which Jamesville Reservoir water/sediment was incubated with ^{14}C-DCB.2HCl in biometer flasks did not show the evolution of $^{14}CO_2$, indicating that the non-recoverable portion of the DCB is adsorbed to sediment. The difficulty

in extraction of DCB from sediment precluded the further use of sediment-fortified water samples because of the inability to recover more than a fraction of the original material.

Microbial Degradation in Activated Sludge --

The fate of DCB in activated sludge was studied because waste treatment systems may represent an important site of entry of DCB into the environment and because the sludge microbial community may have a higher biodegradation capacity than lake water communities. In the samples containing sludge and solids, only 34.7% of the original DCB (analyzed by HPLC) was recovered in the acetonitrile-water and methanol extracts of the non-sterile samples, as opposed to 29.8% in the sterile samples after a 7-day incubation. In the sludge supernatant incubations, 83.6% of DCB was recovered from the non-sterile samples, but only 28.8% from the sterile controls. This difference might be caused by autoclaving, which resulted in a heavy residue, even in the settled supernatant sludge, and possibly changed the adsorptive properties of the solid matter and efficiency of extraction. Carbon-14 values agreed with DCB (as analyzed by HPLC) within 5%, and no materials other than DCB were detected. Less than 0.1% of the ^{14}C incubated in biometer flasks was detected as evolved $^{14}CO_2$ over a 10-day period. Much of the unaccountable ^{14}C appeared to be in the remaining sludge residue, although variable quenching and heterogenous samples prevented an accurate determination of this portion by scintillation counting. It is likely that adsorption to the solids in activated sludge was responsible for the decrease in free DCB.

To minimize the loss of DCB from adsorption on the sludge solids and microorganisms and to determine whether activated sludge microorganisms would adapt to degrading DCB, we examined the biodegradation of DCB in a test system that utilized settled activated sludge as a source of microbes, rather than as an incubation medium and included repeated weekly subcultures into fresh medium.

The results shown in Table 13 show that the subculture enrichment method through weekly subcultures into fresh medium did not increase the biodegradation of DCB. The decrease of DCB seen in this system was comparable to that seen in lake water. As in the previous studies, a considerable amount of the ^{14}C unaccounted for in the analyzed water samples was detected in the organic material formed during incubation. For example, ^{14}C in the isolated organic material of the third culture accounted for 12 to 17% of original ^{14}C in the incubations. Again, no DCB metabolites were detected by HPLC analysis.

Microbial Release of DCB from Yellow Pigment 12 --

The amount of DCB detected in the Yellow Pigment 12 incubations after 28 days was extremely low (Table 14). In addition, the DCB values from the control incubations indicate that the release might be non-biological or the results of a trace DCB impurity in the starting material.

TABLE 13. DEGRADATION OF DCB BY ACTIVATED SLUDGE
(weekly subculture enrichment)

Treatment	Percent DCB remaining after 7 days of incubation		
	DCB (% 0 time)		
	Subculture*		
	0	1	2
Sterile Control	100.6	101.2	99.6
Activated Sludge + 100 mg/l Yeast Extract	84.6	87.3	90.5
Activated Sludge + 50 mg/l Yeast Extract	87.3	73.6	82.5

* Sampling and culturing procedures are explained in Section 4. The data represents one experiment per treatment.

TABLE 14. MICROBIAL RELEASE OF ^{14}C-DCB FROM ^{14}C-YELLOW PIGMENT 12

Sample	^{14}C-DCB-ppb*
Sterile Control	0.12
Muskegon Co. Lagoon	0.14
Jamesville Reservoir	0.16
Oneida Lake	0.06

* Values are the average of two replicates, each analyzed by two different TLC systems.

Discussion

Our findings indicate that DCB is not readily degraded by microorganisms under the experimental conditions. There was some indication of biodegradation in the lake water, although no distinct metabolites were detected. We speculate that the loss of the chemical noticed was primarily the result of adsorption and/or accumulation by microorganisms in the water.

The adsorption of hydrophobic chemicals such as methoxychlor and toxaphene to aquatic microbes has been well-documented (23,24). The affinity of

DCB for aquatic sediments and many organic materials was repeatedly observed in this study. Our results show that, during the course of incubation, a portion of DCB becomes associated with organic matter in the water. The addition of acetonitrile releases or solubilizes the majority of this bound ^{14}C-material, although a portion remains associated with the organic debris. This suggests that the progressive decrease in DCB detected during the incubation period is probably related to the increase of organic material formed during incubation, resulting in a DCB/organic matter complex that cannot be analyzed by chromatographic techniques. In this regard, Wilson (25) showed that incomplete recoveries of DDT from natural water were the result of adsorption to suspended matter in the water. The results of the studies utilizing whole and settled activated sludge indicate that adsorption, rather than metabolism, may predominantly determine DCB fate in an activated sludge unit. No evidence was seen to indicate that sludge microbial populations may be induced to metabolize DCB. This is in contrast to benzidine, which is rapidly degraded in sludge systems (26), even without a period of acclimation.

Even in the event that DCB loss was the result of active microbial degradation, however, the rate of this process is slow. The rate of loss of detected DCB in the lake water systems tested indicates half-lives greater than 40 days, based on the initial rate of DCB loss. In contrast, the half-life of DCB loss in the sediment sorption and photodegradation experiments was measured in minutes. Also, the stability of DCB in sterile samples protected from light indicates that hydrolytic, oxidative, or other chemical mechanisms do not significantly degrade DCB in aqueous solution.

The resistance of Yellow Pigment 12 to microbial metabolism, releasing free DCB, can be attributed in part to its extreme insolubility in water, in which it forms clumped aggregates. It is unlikely that the pigment can be absorbed by microbes for intracellular metabolism. The enzymes necessary for DCB release from the pigment may not be of the extracellular type. Because of the inability to directly measure the amount of pigment in the incubations, we cannot exclude the possibility that DCB was released and subsequently degraded or adsorbed to organic material in the water. Our studies showed, however, that DCB is resistant to microbial metabolism, and the extraction procedures employed should have removed the majority of DCB bound to organic material.

UPTAKE, ELIMINATION, AND METABOLISM OF DCB IN BLUEGILLS

Results

Uptake of DCB --

A preliminary study was conducted to assess the effect of DCB concentration and fish loading ratios upon DCB uptake in a static system. The loss of ^{14}C from the water was monitored for 24 hours, after which time the fish were sacrificed for ^{14}C analysis. The water bathing the fish was also analyzed for DCB and possible metabolites.

As shown in Figure 4, DCB uptake is dependent on both initial DCB concentration and the biomass of fish present. Uptake was initially fast, followed by a relatively slower rate, perhaps indicating the approach of a near-equilibrium in the system. After 24 hours of exposure, an appreciable concentration of ^{14}C-materials was detected in the fish (Table 15), mainly in the non-edible portions. In order to determine whether any metabolites resulting from DCB metabolism in fish were excreted into the water, the nature of the ^{14}C remaining in the water bathing the fish was examined. Essentially, all of the radioactivity in the water was extractable with diethyl ether at pH 10. Thin-layer chromatographic analysis of the ether extract indicated the presence of only one spot with an Rf value corresponding to that of authentic DCB.

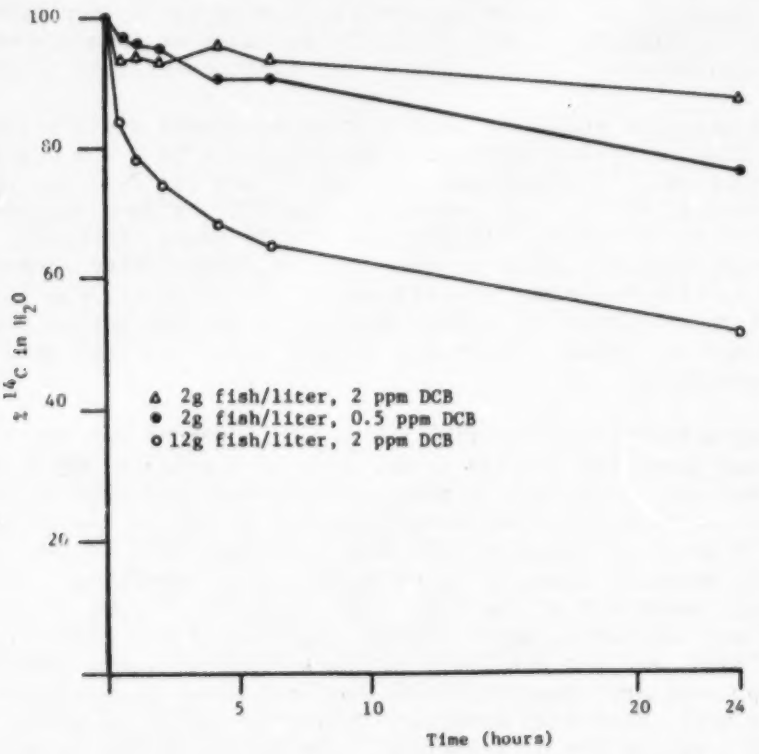

Figure 4. Disappearance of ^{14}C DCB from water in presence of bluegills.

TABLE 15. ^{14}C RESIDUES IN BLUEGILLS AFTER 24 HOURS EXPOSURE TO ^{14}C-DCB.2HCl

	Edible flesh	Head and viscera	Whole fish
2g fish/1, 2 ppm DCB	220.4	448.6	316.8
12g fish/1, 2 ppm DCB	89.5	228.3	149.5
2g fish/1, 0.5 ppm DCB	68.2	215.2	131.3

In subsequent studies, loading ratios of 2 to 3g fish/liter were maintained wherever possible. Continuous-flow exposure experiments were not attempted because of problems entailed in disposal of large volumes of water containing carcinogenic material. In experiments that exceeded 24 hours, the fish were placed in fresh water containing DCB at each 24-hour interval. This step minimized effects on uptake kinetics caused by depletion of DCB in the water bathing the fish. Also, the exchange of exposure water prevented deterioration of water quality that might contribute to fish mortality.

A more detailed study was conducted to determine equilibrium values of DCB and derived materials achieved in fish exposed to 2 ppm DCB.2HCl. As shown in Table 16, ^{14}C uptake was very rapid, and residues in the fish increased through 48 hours of exposure. The ^{14}C-residues were concentrated predominantly in the non-edible portion. By 24 hours, however, an equilibrium was apparently reached in this portion, after which concentration into the edible portion increased significantly. By 48 hours, the fish exhibited symptoms of intoxication and within several hours, the entire remaining test population had succumbed. Control fish held under the same conditions showed no mortality.

Because mortality in the fish exposed to 2 ppm of DCB may have been a result of the inability of fish to detoxify or eliminate DCB at a rate exceeding the rate of uptake, a similar study was conducted at 0.5 ppm to determine ultimate equilibrium concentrations of DCB in bluegills. Through the first 48 hours of exposure, the fish responded well to food and external stimuli and, overall, appeared to be in excellent condition. After 72 hours, however, only about 10% of the test fish responded to food or stimulus. Between 96 and 120 hours, approximately one-half of the remaining test population died, and the survivors exhibited extreme toxic symptoms, as noted in the 2 ppm studies. These symptoms included non-responsiveness (even to the capture net), depressed opercular movements, reddened gills, and hemorrhagic areas on the body surface. The uptake of DCB in this study is shown in Table 17. As in the 2 ppm experiments, residues in the head and viscera fraction appeared to reach near-equilibrium levels prior to the edible flesh fraction. Residue levels in the fish dead at 120 hours were essentially the same as those in the live fish sampled at 96 hours. However, levels in fish surviving through 120 hours were substantially higher.

TABLE 16. ^{14}C DISTRIBUTION IN BLUEGILLS EXPOSED TO 2.0 ppm ^{14}C-DCB.2HCl

	ppm-DCB Equivalent*		
Exposure time (hours)	Edible flesh	Head and viscera	Whole fish
3	92.9 ± 1.9	151.1 ± 2.3	119.2 ± 1.8
6	83.6 ± 3.5	179.1 ± 13.0	123.1 ± 7.3
24	75.6 ± 2.2	368.7 ± 9.5	187.0 ± 3.8
48	192.5 ± 4.9	358.8 ± 6.1	265.3 ± 7.3

*The values are the average of two experiments, with three fish per experiment samples at each exposure intervals. Non-extractable ^{14}C residues were less than 0.5% of total ^{14}C (<0.1 ppm).

TABLE 17. ^{14}C DISTRIBUTION IN BLUEGILLS EXPOSED TO 0.5 ppm ^{14}C-DCB.2HCl

	ppm-DCB Equivalent*		
Exposure time (hours)	Edible flesh	Head and viscera	Whole fish
24	20.5 ± 1.3	98.2 ± 6.4	59.4 ± 2.8
48	39.0 ± 0.3	193.5 ± 13.4	109.7 ± 4.1
72	118.5 ± 6.6	207.1 ± 27.6	164.1 ± 13.7
96	98.3 ± 7.1	212.6 ± 15.3	151.3 ± 10.3
120	213.2 ± 9.4	372.4 ± 29.6	277.0 ± 14.8

*Values are the average of two experiments, with two fish per experiment sampled at each exposure interval. Non-extractable ^{14}C residues were less than 0.5% of total ^{14}C (<0.1 ppm).

Elimination of DCB --

Some of the fish exposed to 2 ppm DCB.2HCl for 24 hours were transferred to water free of the chemical to determine the rate of ^{14}C-residue elimination (depuration). In the 0.5 ppm study, surviving fish at 120 hours were chosen for depuration studies. The fish were placed in fresh water, flowing at a rate to give 12 complete turn-overs of water per 24-hour period. During this period, both water and the fish were periodically sampled and analyzed for ^{14}C content.

The depuration of DCB and derived materials is shown in Tables 18 and 19. Although the rate of elimination is extremely rapid initially, DCB levels in

the fish remained virtually constant after this excretion phase, on a whole fish basis. Also symptoms of toxicity disappeared within several hours of initiating the depuration.

TABLE 18. ELIMINATION OF ^{14}C FROM BLUEGILLS EXPOSED TO 2 ppm ^{14}C-DCB.2HCl

	Percent of initial ^{14}C remaining*		
Depuration time (hours)	Edible flesh	Head and viscera	Whole fish
24	45.6 ± 3.2	51.5 ± 0.7	42.2 ± 7.4
48	37.2 ± 4.4	42.3 ± 8.0	33.6 ± 2.1
72	35.1 ± 4.1	34.8 ± 11.7	28.9 ± 0.2

*Initial ^{14}C residues were 60.7 ppm, 233.7 ppm, and 170.4 ppm for edible flesh, head and viscera, and whole fish, respectively (average of 3 experiments). Depuration data are the average of duplicate experiments (4 fish per experiment).

TABLE 19. ELIMINATION OF ^{14}C FROM BLUEGILLS EXPOSED TO 0.5 ppm ^{14}C-DCB.2HCl

	Percent of initial ^{14}C remaining*		
Depuration time (hours)	Edible flesh	Head and viscera	Whole fish
24	-	-	56†
120	5.2 ± 0.3	14.4 ± 0.1	11.6 ± 0.2
240	2.6 ± 0.1	19.7 ± 0.1	13.3 ± 0.1

*Initial ^{14}C residues were 213.2 ppm, 372.4 ppm, and 277.0 ppm for edible flesh, head and viscera, and whole fish, respectively (average of 2 experiments). Depuration data are the average of duplicate experiments (4 fish per experiment).
†Calculated from the ^{14}C in depuration water over the first 24 hours of the experiment.

Metabolism of DCB --

Preliminary evidence of metabolism of DCB by fish was obtained by TLC of methanol extracts of the edible flesh or head and viscera fractions. Chromatography in two solvent systems showed the presence of at least two materials. One radioactive peak co-chromatographed with DCB whereas another remained at the origin, indicating that it was of a polar nature. To confirm this possibility, the fish extracts were fractionated according to partitioning behavior. The ^{14}C material that partitioned into ether from basic

(pH 11) water (fraction A) was found to contain only DCB as shown by TLC. The water-soluble material remaining (fraction B) did not partition into ether when the aqueous medium was acidified to pH 1 with HCl. Because authentic DCB is almost quantitatively recovered into ether from basic water in a one-step partition (Appendix 1), the water-solubility of the metabolic derivative might be attributed to a zwitterionic nature (such as hydroxylated products), or the conjugation of DCB to a material that retains a free acidic functional group and liberates free DCB upon exposure to mildly acidic conditions. The latter possibility was investigated by rebasification and partitioning of the acid-treated aqueous phase with ether. The ^{14}C was quantitatively recovered into ether, and co-chromatographed with DCB in two thin-layer chromatographic systems. This indicates that a highly acid-labile conjugate of DCB is formed in the fish. The conjugate was hydrolyzed to form free DCB in as little as 30 seconds at pH 1, but was stable at pH 9 or higher. The identity of the fraction A material and the material produced by acid treatment of fraction B was further confirmed to be DCB, both qualitatively and quantitatively, by HPLC. In addition to identical retention times, uv absorbance of the eluted unknown materials was scanned with the chromatograph uv detector. The uv spectra of both samples was virtually the same as authentic DCB, including identical λ max. = 282 nm. The identification procedures and results are summarized in Figure 5. Therefore, the degree of metabolism seen in the uptake and elimination studies is reported as the distribution of extracted materials into the fraction A (DCB), and fraction B (metabolite) as determined radiometrically.

The partition behavior of the metabolite in basic aqueous media indicates that the DCB nucleus was modified by addition of at least one ionizable acidic group. Mono- or bis-N-sulfate or N-glucuronide conjugates of DCB would be expected to behave in such a manner. Because of the limited amounts of free sulfate available in vertebrate systems, sulfate conjugates rarely constitute a major portion of metabolism of foreign chemicals (27). In contrast, glucuronidation is well-documented as an important metabolic pathway in vertebrates, including fish (28). Futhermore, previous studies of aromatic amine metabolism indicates the occurrence of N-glucuronidation (29). These conjugates are highly labile under mildly acidic conditions (30) and may be formed in the absence of the enzymes required for synthesis of O-glucuronide conjugates (31).

To check this possibility, DCB was mixed with glucuronic acid according to the procedure of Boyland et al. (32). The crystalline product obtained was washed with ethanol and dried. The synthetic material obtained was stable in pH 10 media, exhibiting a λ max. of 285 nm (as opposed to 282 nm for DCB under identical conditions). When the pH was adjusted to 2 with HCl, a maximum absorbance at 248 nm (corresponding to DCB) rapidly appeared. The acid-lability of the synthesized product, producing free DCB, was confirmed by both thin-layer and high performance liquid chromatography. Without the acid treatment, both the synthetic and biological materials appeared to elute with the column void volume. Bovine liver β-glucuronidase (P-L Biochemicals, Milwaukee, WI) was inactive in catalysis of the conjugate, relative to non-enzymic controls (pH 7 and 9). The inactivity of β-glucuronidase towards N-glucuronide conjugates of aromatic amines has been noted previously (30).

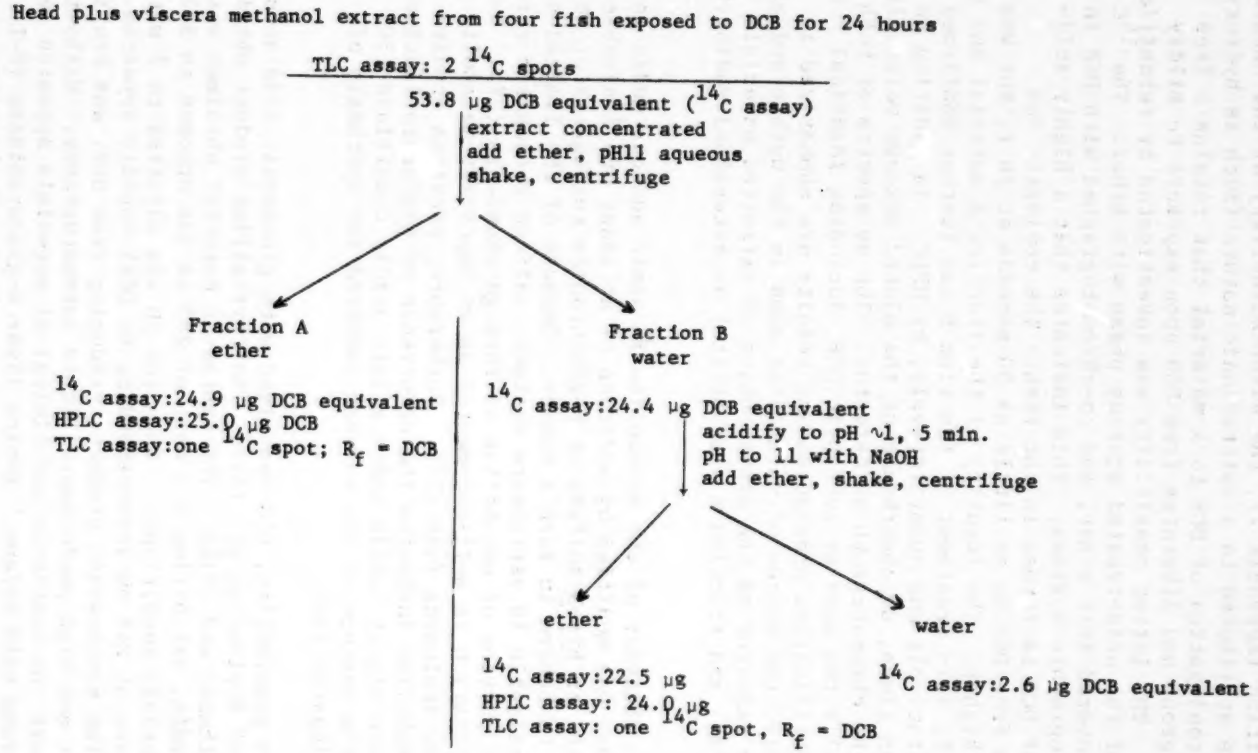
Head plus viscera methanol extract from four fish exposed to DCB for 24 hours
TLC assay: 2 14C spots
53.8 µg DCB equivalent (14C assay)
extract concentrated
add ether, pH11 aqueous
shake, centrifuge
Fraction A
ether
14C assay:24.9 µg DCB equivalent
HPLC assay:25.0 µg DCB
TLC assay:one 14C spot; Rf = DCB
Fraction B
water
14C assay:24.4 µg DCB equivalent
acidify to pH ∿1, 5 min.
pH to 11 with NaOH
add ether, shake, centrifuge
ether
14C assay:22.5 µg
HPLC assay: 24.0 µg
TLC assay: one 14C spot, Rf = DCB
water
14C assay:2.6 µg DCB equivalent

Figure 5. Fractionation of fish tissue extract for DCB and metabolite verification.

As shown in Tables 20 and 21, the amount of metabolite present was dependent on both time and DCB concentration, indicating a rate-limiting factor in biosynthesis. This might be a limited amount of conjugating material (such as glucuronic acid), and/or that the rate of biosynthesis is less than the rate of DCB uptake, particularly in the early stage of exposure. Relatively less metabolite is present at 24 hours in the 2.0 ppm exposures than at 0.5 ppm. Within 48 hours, an equilibrium between the relative amounts of DCB and metabolite in the edible and non-edible fractions appears to have been reached, except that metabolite in the edible fraction of fish exposed to 2.0 ppm DCB may be increasing at this point. This equilibration is probably reached through stabilization of factors including rates of DCB uptake and elimination, and conjugate synthesis, hydrolysis, and elimination. Distributional mechanisms between edible and non-edible tissues probably contribute as well.

Discussion

Dichlorobenzidine is bioaccumulated by fish to considerable levels, apparently limited by the toxicity of the chemical. This is indicated by the presence of nearly equal levels of ^{14}C residues in both the 2.0 and 0.5 ppm exposures (approximately 250 ppm on a whole fish basis) at the point at which the experiments were terminated due to mortality. Except for a brief period of stabilization in the 0.5 ppm exposures, residues increased through the course of the experiments. The high residue levels seen in fish surviving at 120 hours in the 0.5 ppm exposures, compared with the 96-hour samples, might be due to the fact that mortality reduced the biomass of the systems, resulting in a greater availability of DCB on a per fish basis. Also, the fish surviving at 120 hours represent a biased sample in the sense that survival might be the result of metabolic and/or physiological factors unique to the survivors that, in turn, allowed higher concentrations of residues to be achieved. This effect could have been masked when random samples of the general test population were taken at previous time intervals. It should be reiterated that the surviving fish were severely intoxicated and would have succumbed if exposure had been continued beyond 120 hours.

The nature of elimination of DCB from contaminated fish after transfer to fresh water was different from many previous observations. Other lipid-soluble chemicals such as lindane and methoprene are bioconcentrated to a large degree but are progressively eliminated after removal to non-contaminated water (33,34). In this investigation, however, an extremely rapid initial rate of elimination was followed by a very low or negligible rate, with appreciable residues, primarily in the non-edible portions, remaining even after 10 days of exposure to fresh water. This observation suggests the possibility of an enterohepatic circulation of DCB and metabolite or that a portion of the residues are tightly bound to lipoproteins or other substances and resistant to elimination. The former possibility is indicated by the preponderance of conjugated DCB in the non-edible fraction of the fish after the rapid elimination phase has ceased. The likelihood of biliary excretion of a large molecule such as the DCB conjugate, plus the labile nature of this material under acid conditions, such as found in the digestive tract, also favor this event.

TABLE 20. RELATIVE ABUNDANCE OF DCB AND METABOLITE IN BLUEGILLS EXPOSED TO 2.0 ppm ^{14}C-DCB.2HCl

	Percent abundance*											
	Edible flesh				Head and viscera				Whole fish			
Exposure (hours)	A		B		A		B		A		B	
3	94.4	(87.7)	5.6	(5.2)	85.5	(129.2)	15.5	(23.4)	88.8	(105.8)	11.2	(13.4)
6	93.8	(78.4)	6.2	(5.2)	86.3	(154.6)	13.7	(24.5)	89.5	(110.2)	10.5	(12.9)
24	70.2	(53.1)	29.8	(22.5)	59.0	(217.5)	41.0	(151.2)	61.9	(115.8)	38.1	(71.2)
48	59.8	(115.1)	40.2	(77.4)	60.5	(217.1)	39.5	(141.7)	60.2	(159.7)	39.8	(105.6)
Depuration (hours)												
24	35.2	(16.1)	64.8	(29.5)	28.4	(14.6)	71.6	(36.9)	21.0	(8.9)	79.0	(33.5)
48	28.2	(10.5)	71.8	(26.7)	21.3	(9.0)	78.7	(33.3)	23.0	(7.7)	77.0	(25.9)
72	27.5	(9.6)	72.5	(25.4)	26.6	(9.3)	73.4	(25.5)	26.4	(7.6)	73.6	(21.3)

*Values are the average of duplicate analyses of 6 fish pooled for analysis. The values in parentheses are the data expressed as ppm-DCB equivalent.

A - Refers to DCB
B - Refers to the acid-labile conjugate of DCB

TABLE 21. RELATIVE ABUNDANCE OF DCB AND METABOLITE IN BLUEGILLS EXPOSED TO 0.5 ppm ^{14}C-DCB.2HCl

	Percent abundance*											
	Edible flesh				Head and viscera				Whole fish			
Exposure (hours)	A		B		A		B		A		B	
24	37.1	(7.6)	62.9	(12.9)	18.0	(17.7)	82.0	(80.5)	21.3	(12.7)	78.7	(46.7)
48	42.2	(16.5)	57.8	(22.5)	22.3	(43.2)	77.7	(150.3)	26.1	(28.6)	73.9	(81.1)
72	50.9	(60.3)	49.1	(58.2)	43.3	(89.7)	56.7	(117.4)	46.0	(75.5)	54.0	(88.6)
96	45.8	(45.0)	54.2	(53.3)	42.3	(89.9)	57.7	(122.7)	43.5	(65.8)	56.5	(85.5)
120	45.2	(96.4)	54.8	(116.8)	39.2	(146.0)	60.8	(226.4)	41.9	(116.1)	58.1	(160.9)
Depuration (hours)												
120	51.2	(2.7)	48.8	(2.5)	29.2	(4.2)	70.8	(10.2)	33.1	(3.8)	66.9	(7.8)
240	28.5	(0.7)	71.5	(1.9)	13.0	(2.6)	87.0	(17.1)	14.2	(1.9)	85.8	(11.4)

*Values are the average of duplicate analyses of 4 fish pooled at each time intervals. The values in parentheses are the data expressed as ppm-DCB equivalent.

A - Refers to DCB
B - Refers to acid-labile conjugate of DCB

The identity of the acid-labile conjugate was not directly determined because of inability to recover the material intact into non-aqueous media. In all aspects examined, the material showed properties similar to the synthetic N-glucuronide of DCB and other aromatic amines (29). These properties include a free acidic functional group, high lability under acidic conditions, chromatographic behavior, and resistance to hydrolysis by β-glucuronidase. The ease of synthesis of the conjugate may indicate that the reaction proceeds non-enzymatically *in vivo*. It is unlikely that the product is an artifact caused by reaction of DCB during the extraction procedures, because the relative and absolute amounts increase with time of exposure.

No evidence of alteration of the DCB nucleus, such as ring hydroxylation, was found in this study. Hutzinger *et al.* (35) did not detect hydroxylation in the metabolism of a series of polychlorinated biphenyls by brook trout, although a hydroxylated product was found to be a minor metabolite of tetrachlorobiphenyl in rainbow trout (36). Although microsomal enzymes catalyzing hydroxylation and other oxidative modifications of foreign chemicals are known to occur in fish (28), *in vivo* importance of these enzymes may be less in fish than in mammals, because of the much smaller relative size of the liver (the major site of these enzymes) and the lower temperatures involved.

Equilibrium bioconcentration factors (based on total ^{14}C residue) of 38 to 44 in edible flesh were reported in a study of the uptake of benzidine in bluegills (37). Levels were 12- to 43-fold higher in the non-edible portions. No evidence of toxicity was seen. Elimination of residues was progressive in the depuration phase of the studies, with 70 to 73% of the residues in the edible portion and 96% of those in the non-edible portions eliminated after 14 days exposure to fresh water. There appears to be a higher concentration into the non-edible portions achieved by benzidine than dichlorobenzidine, although concentration into edible portions was much higher for DCB. The lack of toxic symptoms seen for benzidine might be the result of the continuous-flow exposures utilized. It is possible that static conditions potentiated DCB toxicity through accumulation of waste products. It is equally likely, however, that DCB is more toxic to fish than benzidine. The action of toxicants is often well-correlated to lipophilicity and water solubility (38). This situation may apply to the benzidine series as well, as dichlorobenzidine is much less water soluble and more lipophilic than benzidine.

Bioconcentration factors in fish of greater than 10^4 have been recorded for such chemicals as DDT (39) and Arachlor 1254 (40), under equilibrium conditions. In comparison, the bioconcentration factor for DCB of 132 to 554 (for DCB and its conjugate) are rather low. The DCB values, however, do not represent equilibrium values and could potentially have been higher if mortality had not occurred. Also, the presence of even relatively low concentrations of DCB must be regarded as significant in view of the carcinogenic hazard posed by the chemical.

SECTION 6

GENERAL DISCUSSION

Based on the results of this study, it is apparent that physico-chemical processes will largely determine the persistance of DCB in the aquatic environment and, ultimately, the associated health and ecological hazards. The degree of adsorption to sediments and other suspended matter may dictate the ultimate fate of DCB in aquatic environments. For example, sorption of a chemical may have a negative effect upon rates of biodegradation and photodecomposition. Sorption may also lead to a wide translocation of the chemical from its original site of entry, and possibly to accumulation through trophic levels by the action of detritus-feeding organisms. In contrast, sorption may serve as a mechanism of DCB inactivation if the affinity between the sediment and chemical is sufficiently strong. In the case of DCB, further research is required to determine the ultimate role of sorption mechanisms in the fate of DCB. The difficulty in extraction of more than a fraction of sorbed DCB suggests that very strong interactions, perhaps covalent, are involved. By analogy with chloroanilines and other amines, incorporation of DCB into humic-like materials might serve as a major means of DCB degradation. The affinity of DCB for organic materials also dictates that the adsorption to biological entities such as microbes and plankton be assessed because bioaccumulation of DCB by this route might lead to a direct threat to human health.

In a similar fashion, the photodegradation of DCB presents a paradoxical situation. Ultimately, the photolysis of DCB may lead to innocuous products. However, in the process, monochlorobenzidine and benzidine are generated, which may pose a greater carcinogenic hazard than the parent chemical. The degree of photolysis in a natural aquatic system is dependent on many factors; depth, turbidity, latitude, season, and climate (41). As a result, the ultimate role of photolysis must be assigned on a case by case basis.

Compared to sorption and photolysis, biodegradation appears to be a minor component governing the fate of DCB. Hydroxylation of the ortho position of benzidine appears to be the major site of transformation of that chemical. It may be that substitution of the benzidine nucleus with chlorine at the 3-carbon positions decreases the susceptibility of the molecule to enzymatic attack.

The release of DCB from DCB-based pigments does not appear to be an important source of entry of DCB into the environment. This process may be more important with water-soluble dyes.

DCB is bioconcentrated by fish to a significant degree directly from contaminated water. Toxic levels of the chemical may be achieved, although further studies at lower concentrations are indicated. No evidence of direct metabolism of the DCB nucleus was seen. However, the fish are capable of conjugating DCB. This conjugate, apparently an N-glucuronide, is labile to hydrolysis under very mildly acidic or neutral conditions and, therefore, should be considered the toxicological equivalent of DCB. In addition, a portion of the DCB in contaminated fish, mainly situated in the non-edible portions, remains within the fish after placement in a non-contaminated environment, giving an indication of a long residual life of DCB in higher life forms.

As a final note, workers involved in monitoring DCB in the environment should consider the photolability and high affinity for organic matter displayed by DCB. Exhaustive extractions are indicated, and benzidine and monochlorobenzidine should be monitored as well.

Future work designed to determine the hazard potential of DCB should focus upon the relative contributions of these several mechanisms governing DCB fate as they operate in concert.

APPENDIX

WATER SOLUBILITY AND PARTITION ANALYSIS OF DCB

Water Solubility of DCB

The solubility of DCB.2HCl in aqueous buffers of pH 4.6-8.9 at 22 ± 1° was determined spectrophotometrically from a knowledge of the extinction coefficients of its uv absorption maxima. All experiments were conducted in the dark or with glassware wrapped with aluminum foil to exclude light. To measure extinction coefficients, a weighed amount of DCB.2HCl (typically 5 to 30 mg) was introduced into a 50- or 100-ml volumetric flask, shaken with methanol and made up to the mark. Aliquots (0.1 to 1 ml) were pipetted out, introduced into volumetric flasks of 25 to 100 ml capacity, concentrated to about 1.1 ml by a stream of air and made up to the mark with the appropriate buffer. The uv spectra of these solutions were recorded, and the values derived from at least two separately weighed methanol solutions, and from 4 to 6 spectral determinations.

An excess of DCB.2HCl was mechanically shaken with the appropriate buffer for up to 7 days. Samples were withdrawn at daily intervals beginning from 10 hours after mixing and were centrifuged at 17,000 rpm for 30 minutes, and the uv spectra were then recorded.

Recovery of DCB from Water

To determine the recovery of DCB from water with organic solvents, aqueous solutions of 2 ppm ^{14}C-DCB.2HCl were shaken vigorously with equal volumes of organic solvent and centrifuged. The distribution of ^{14}C in the phases was measured by scintillation counting.

Results

Water Solubility of DCB --

Ultraviolet spectra of the surface layer of the supernatant liquid showed essentially no change in DCB concentration over the 7-day period. In separate experiments, DCB.2HCl was warmed at 60°C with the appropriate buffer, cooled to ambient temperature and worked up as above. The uv spectrum of the centrifuged solution indicated that supersaturation to the extent of about three times the solubility at room temperature had occurred. Furthermore, the solution remained stable for several hours and returned to the solubility limit only after it was mechanically shaken overnight. The spectral and solubility data are listed below. The similarity of the solubility results

is not surprising since DCB remains in its free base form throughout the pH range studied (the first pK_a of benzidine is 4.66, and that of DCB is therefore lower). The variation in solubility in the three media is possibly a consequence of salt effects.

Spectral and Solubility Data of DCB.2HCl at 22°C

pH*	E (λnm)		E (λnm)		Solubility (ppm)†	
8.9	26,200	2% (282)	53,600	0.4% (212)	4.59	4%
6.9	28,200	1% (282)	57,800	1% (211)	3.99	2%
4.6	27,000	4% (283)	52,000	4% (212)	5.36	4%

*These refer to borate (8.9), phosphate (6.9) and acetate (4.6) buffers.

†Averaged from at least 4 determinations. Calculations are based on the peak at 282 nm. Results based on the maximum at 212 nm give essentially identical results.

Recovery of DCB from Water

The partition coefficients obtained are listed below. The data show that DCB partitioned into organic solvents is pH dependent and efficient at pH 7 or greater. Based on the pH-dependence of partition of DCB into ether reported by Glassman and Meigs (42), the first and second pK_a of DCB are less than 4. In general, extraction of DCB from aqueous media was performed after adjusting pH to 10, to permit detection of transformation products which might also partition into ether at intermediate conditions of pH. The organic solvent partition coefficients obtained are lower than the distribution coefficients obtained from aquatic sediments (Section 5). This difference may relate to the solvents used, since n-octanol is often preferred for correlating partition properties to biological or environmental events. Also, the above determinations were done primarily to test DCB extraction from water, using ^{14}C-DCB of low specific activity (relative to sediment partition experiments). Therefore, the partition coefficients probably represent minimum values since the difference between an observed K_p of 199 (99.5% recovery into organic solvent under the experimental conditions) and a K_p of 499 (99.8% recovery), for example, represents only a very small difference in measured radioactivity which lies within the range of experimental error, particularly in measuring the ^{14}C remaining in the aqueous phase.

Recovery of DCB from Water

Phase		K_p*
Organic	Aqueous	
benzene	pH 11	124†
ether	pH 11	199
ether	pH 7	199
ether	pH 1	0.04
hexane	pH 11	82.3

†Data are the average of triplicate determinations.

*K_p = [DCB] organic/[DCB] aqueous

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